MANGALORE UNIVERSITY

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"Studies on TNF-alpha as a therapeutic biomarker in the management of obesity induced diabetes"

Ph.D. FINAL SYNOPSIS

Submitted to Department of Biochemistry
Mangalore University

FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

By

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MU/EXB/PhD/CR.67/Bio-chem./2015-16/E-13

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Jun 2022

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ABBREVIATIONS

%	:	Percentage
µl	:	Microliter
bp	:	base pair
cDNA	:	Complementary DNA
CNS	:	Central Nervous System
DCFH-DA	:	2′,7′-Dichlorofluorescin diacetate
DM	:	Diabetes Mellitus
DNA	:	Deoxyribonucleic Acid
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
ELISA	:	Enzyme Linked Immune Sorbent Assay
FACS	:	Fluorescence-Activated Cell Sorting
FBS	:	Fetal Bovine Serum
FFA	:	Free Fatty Acid
GLP-1	:	Glucagon-Like Peptide 1
GLUT4	:	Glucose transporter type 4
GO	:	Gene Ontology
HAT	:	Hypoxanthine and Thymidine
HFD	:	High Fat Diet
HFD+PE	:	High Fat Diet + Plant Extract
HFHS	:	High Fat High Sugar
HIS	:	Hyper Immune Sera
HQC	:	High Quality Control
HRP	:	Horseradish Peroxidase
IC50	:	Inhibitory Concentration at 50 %
IFNγ	:	Interferon gamma
IgG	:	Immunoglobulin G
IL-17	:	Interleukin 17
IL-1B	:	Interleukin 1 Beta
iNOS	:	Inducible Nitric Oxide Synthase
IR	:	Insulin Resistance
IR-A	:	Insulin Receptor-A
IR-B	:	Insulin Receptor-B
IRS-1	:	Insulin Receptor Substrate 1
IRS2	:	Insulin receptor substrate 2
kDa	:	Kilodalton
KEGG	:	Kyoto Encyclopedia of Genes and Genomes
Kg	:	Kilogram
LLOQ	:	Lower Limit of Quantification
LQC	:	Low Quality Control
M	:	Molar
MAPK	:	Mitogen-Activated Protein Kinase
MCP-1	:	Monocyte Chemoattractant Protein-1
mg	:	miligram
mg/dl	:	milligrams per decilitre

mM	:	milimolar
MQC	:	Middle Quality Control
mRNA	:	messenger Ribonucleic Acid
N	:	Normality
ND	:	Normal Diet
NiNTA	:	Nickel Nitrilotriacetic Acid
nm	:	nanometer
PCR	:	Polymerase Chain Reaction
PE	:	Plant Extract
pH	:	potential of Hydrogen
PI(3)K	:	Phosphoinositide 3-Kinases
PIS	:	Pre immune Sera
PKC	:	Protein kinase C
QC	:	Quality Control
RIN	:	RNA Integrity Number
RNA	:	Ribonucleic Acid
ROS	:	Reactive Oxygen Species
RPM	:	Revolutions Per Minute
SD	:	Standard Deviation
SDS-PAGE	:	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SGLT2	:	Sodium-Glucose Transport Protein 2
STZ	:	Streptozotocin
T2D	:	Type 2 Diabetes Mellitus
TNF alpha	:	Tumour Necrosis Factor alpha
TZD	:	Thiazolidinediones
ULOQ	:	Upper Limit of Quantification
WAT	:	White Adipose Tissue

CHAPTER 1- INTRODUCTION

The biggest public problem in the 21st century, causing enormous burden on the economy, health and quality of life worldwide is diabetes. Among diabetes, Type 2 diabetes accounts for a large majority of diabetic cases, occurrence of which is closely associated with the development of insulin resistance (IR) accounting for 90 % of diabetic cases(Liu *et al.*, 2017). Within Karnataka 9.4-10 % of the population are diabetic. With this phase of new cases getting added every year, by the year 2024 India will have up to 84% of its population with diabetes. Multiple clinical studies have demonstrated early onset of insulin resistance as a risk factor for developing diabetes and cardiovascular complications at a later stage in life. The excess of circulating triglyceride (TG) due to high caloric intake and free fatty acids (FFA) results in the accumulation of activated lipids in the muscle, (Guilherme *et al.,* 2008) disrupting functions such as mitochondrial oxidative phosphorylation and insulin-stimulated glucose transport, thus triggering insulin resistance(Guilherme *et al.,* 2008). FFA also induced overproduction of superoxide (Giacco and Brownlee, 2010). All these reduces insulin-stimulated phosphorylation of IRS2 (Insulin receptor substrate 2) and IRS2-associated PI (3)K activity through protein kinase-C epsilon (PKCε) activation. High-calorie diet induces low grade chronic inflammatory state, the 'metaflammation' (Forsythe *et al.,* 2008) can be considered as a defensive reaction, yet acts as a double-edged weapon contributing to the multiorgan dysfunction that results in increased insulin resistance as well as in impaired insulin secretion (Kuryłowicz and Kózniewski, 2020). The proinflammatory state that accompanies the metabolic syndrome associates with both insulin resistance and endothelial dysfunction, providing a connection between inflammation and metabolic processes which is rightly termed as meta-inflammation. This meta-inflammatory state affects oxidative stress and lipid content(Volpe *et al.,* 2018).

Current approved-medications include Insulin, Metformin, DPP4-inhibitors, TZDs, SGLT2 inhibitors and GLP-1 receptor agonists. None of the medications decrease the root cause of oxidative stress or insulin resistance.

There are both *in vitro* and *in vivo* models used for diabetic studies. Both have their own advantages and disadvantages. *In vivo* models are either streptozotocin (STZ), alloxan or high fat diet induced diabetic models. These models mimic closely the actual disease condition and can be directly co-related with the severity.

High fat high sugar (HFHS) diet induced models are closer to the reality as that is the main reason for type 2 diabetes. HFHS model is close to the life style induced hyperglycemia (Khan *et al.,* 2019). HFHS feeding is detrimental because high rates of FA oxidation in skeletal muscle can lead to the build-up of toxic metabolites of fat metabolism and the accumulation of pro-

inflammatory cytokines, which further exacerbate the insulin resistance. Overactivation of ubiquitin proteasome pathway, oxidative stress, myonuclear apoptosis, and mitochondrial dysfunction are some of the mechanisms involved in muscle atrophy induced by obesity or in mice fed with HFHS diet (Rasool *et al.,* 2018). *In-vivo* models are limited with the need of animal facility, animal handling personnel and animal ethics. One disadvantage with chemically induced diabetes is that the chemicals can be toxic at other organs of the body. Hence HFHS diet induced models are near to the real-life case and have less side effects.

In vitro models for diabetes can be induced by various ways (Lo *et al.,* 2013)high glucose causing, dexamethasone, TNF alpha, palmitate and hypoxia. Even though these, *in vitro* model does not mimic closely to the actual disease condition they are useful for the molecular studies, more variety of experiments can be performed, no ethical clearance is required. Cellular mechanisms involved in diabetes can be studied using different cell lines.

Anti-diabetic herbal formulations prepared from medicinal plants are popular choices in many countries due to easy access and possibly lesser side effects. In Indian traditional system of medicine, many such plants have been used for their glucose-lowering properties (Tota *et al.,* 2013; Parikh *et al.,* 2014). *Holarrhena antidysenterica* is one of the wonder plants belonging to the family Apocynaceae (Mrinal and Navjeet Singh, 2018) commonly known as "*Kutaja*". This was used initially for the treatment of dysentery as the name comes from. The plant is a native of India and is profound in Sub-Himalayan tract extending from Uttar Pradesh to Meghalaya and in central and peninsular states of Andhra Pradesh, Karnataka, Kerala, Odisha, Tamil Nadu and West Bengal(Jamadagni *et al.,* 2017a). In Ayurvedic and Unani system of medicine it is used in the treatment of diabetes, diarrhea, and skin diseases. Later on, this plant is being explored for its uses in treating many more disease like cancer, CNS related disorders, various pathogenic infections etc., In recent years, various phytochemicals have been isolated from the plant and traditional pharmacological activities are attributed to the phytochemicals present(Mrinal and Singh, 2018).

New finding links diabetes and high fat-high sugar diets with changes in immune cells and inflammatory status, highlighting an emerging realization that diabetes is an inflammatory disease. Drugs designed to block certain inflammatory proteins, can prevent, and treat type 2 diabetes and its complications. It appears that once we control the inflammation, we can begin to get everything else in line(Tsalamandris *et al.,* 2019). Fortunately, there are some inflammatory markers already identified which is a promising compound that seems to work. Three major cytokines implicating in the inflammation of diabetes, are the synergistic action of interferon gamma (IFN-gamma), TNF-alpha and IL-1beta. The combined action of these inflammatory molecules results in the upregulation of inducible nitric oxide synthase (iNOS), with subsequent

production of nitric oxide (NO). However, ROS plays a role in beta cell destruction. Visceral white adipose tissue (WAT) is the major source of inflammatory markers in T2D, is also a target of the inflammatory process in people with diabetes. Further infiltration of adipose tissue by macrophages and immune cells (B cells and T cells) trigger local and systemic chronic low-grade inflammation, by producing more cytokines and chemokines that serve as a pathologic link between obesity, insulin resistance and diabetes (Tsalamandris *et al.,* 2019). TNF-alpha affects insulin mediated glucose uptake at multiple points. Excess TNF-alpha induces serine phosphorylation of IRS-1 preventing tyrosine phosphorylation of the insulin receptor interfering with insulin signal transduction, leading to insulin resistance by preventing the conversion of PIP2 to PIP3, hence inhibiting AKT mediated GLUT4 translocation to the plasma membrane (Emanuelli *et al.,* 2001; Rui *et al.,* 2002; Dandona *et al.,* 2005). In lean condition the adipocytes are small, triglyceride, fatty acid and glucose metabolism is happening normally. Upon increase in weight described as overweight condition the adipocytes enlarge. Due to prolonged over weight condition pro-inflammatory proteins like MCP-1 etc., are secreted by adipocytes themselves which will start infiltration of macrophages which brings in TNF alpha. At this point inflammation and insulin resistance are established, when triglyceride, fatty acid and glucose metabolism are inhibited (Guilherme *et al.,* 2008). Administration of exogenous TNF alpha to animals induce insulin resistance, whereas neutralization of TNF alpha improves insulin sensitivity. The absence of TNF alpha partially protects against obesity-induced insulin resistance in mice. Multiple mechanisms have been suggested to account for these metabolic effects of TNF alpha. TNF alpha is mainly produced in adipocytes and/or peripheral tissues, and induces tissue-specific inflammation through the involvement of generation of ROS and activation of various transcriptional mediated pathways (Hamid *et al.,* 2017).

Anti-TNF alpha treatment strategies have been developed to reduce the incidence of insulin resistance and development of T2DM (Hamid *et al.,* 2017). Commercially available monoclonal antibodies against TNF alpha like Etanercept against autoimmune diseases, Infliximab for the treatment of autoimmune diseases includes Crohn's disease, ulcerative colitis, rheumatoid arthritis, ankylosing spondylitis, psoriasis, psoriatic arthritis, and Bechet’s disease, Adalimumab used in the treatment of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, ulcerative colitis, plaque psoriasis, hidradenitis suppurativa, uveitis, and juvenile idiopathic arthritis and Rituximab for the treatment of non-Hodgkin's lymphoma are already in use, when checked for the diabetic markers in these patients, they showed improvement in the disease condition of diabetes too.

The current therapeutical options that are available for the treatment of diabetes only serve to slow the progression of diabetic complications. New biomarkers and therapies are urgently

needed to control the secretion of cytokines. Several diverse steps along the metabolic signaling route of hyperglycemia-induced diabetes could potentially be considered as the new therapeutical targets(Volpe *et al.,* 2018). Keeping the above in view and considering the increased incidence of diabetes worldwide with limited treatment strategies, we proposed to conduct a study in management of the disease by targeting TNF-alpha and to explore the anti-diabetic activity of *Holarrhena antidysenterica*.

In the study two problem statements are identified and tried to answer.

Diabetes is an inflammatory disease caused by a chronic inflammatory state in the tissues with the increased inflammatory cytokines being secreted. We are targeting a specific inflammatory cytokine like TNF alpha in stopping further progression on chronic inflammation into diabetes.

Experimental evidence has shown that natural compounds used in various therapeutic systems have promising potential as anti-diabetic agents due to array of secondary metabolites having diverse biological effects including antioxidant properties and anti-lipogenesis effect. Can we target different complexities of diabetes like oxidative stress and lipogenesis in controlling insulin resistance?

- ## AIM OF THE STUDY

To ascertain association of TNF-alpha and obesity induced diabetes and develop TNF-alpha inhibitors to prevent obesity induced diabetes.

- ## OBJECTIVES OF THE STUDY

- To identify the role of TNF alpha as a biomarker in diet induced obese mice by *in vivo* studies.
- To test the effect of *Holarrhena antidysenterica* extract in blocking of TNF-alpha in diabetes induced animals.
- To make anti-TNF alpha monoclonal antibody.
- To block TNF-alpha by TNF-alpha inhibitors (anti-TNF alpha antibody and extract from *Holarrhena antidysenterica*) to evaluate the onset of diabetes.
- To check the mechanism of action of TNF alpha inhibitors by genomic studies.
- To verify the TNF alpha pathway emerging from the genomic study by *in vitro* studies.

CHAPTER 2 - REVIEW OF LITERATURE

The recently published literature reports relevant to the present study are discussed in the chapter. This chapter also deals with the information given in the introduction providing the background of insulin resistance in diabetes and experimental procedures for the *in vitro* and *in vivo* diabetic model development; testing anti-TNF alpha antibody and extracts of *H.*

antidysenterica on these diabetic models. The literature survey on an antioxidant and anticancer activity from different parts of *H. antidysenterica* and their importance in the field of diabetes has also been discussed.

CHAPTER 3 - MATERIALS AND METHODS

This chapter is divided into three sections. Instruments and chemicals used for this research work are given in section 1 and 2. In section 3 the detailed methods used for collection of plants, preparation of extracts, preliminary phytochemical screening, and quantification, checking the antioxidant property of the extracts by DPPH method. Procedure for making TNF alpha protein and raising polyclonal and monoclonal antibody against TNF alpha protein. Detailed methodology for sandwich ELISA development. Method used for establishing *in vivo* and *in vitro* diabetic models. Method used to establish an insulin resistant model with impaired lipogenesis showed by Oil-O-Red staining, and ROS production by FACS staining. The methodology employed for RNA sequencing was modified Illumina TruSeq RNA Sample Preparation V2 protocol is also described in detail.

MAKING TNF ALPHA PROTEIN

The mouse TNF alpha extracellular region was codon optimized for *E. coli* and synthesized in pUC 18 vector. The cDNA was PCR-amplified and subcloned into pET28a+ vector between HindIII and Xho I restriction sites. The construct was sequenced to confirm the presence of the mouse TNF cDNA. Further it was expressed in *E. coli* BL21(DE3) cells with kanamycin as a selection pressure.

PURIFICATION OF TNF ALPHA PROTEIN

Purification protocol was carried out as described by Graslund *et al.*, (2008) with a few modifications. The cells were pelleted down by centrifuging them at 8,000 rpm for 10 minutes at RT. Supernatant was discarded and pellet was dissolved in lysis buffer (Tris Cl -20 mM, NaCl-300 mM, Glycerol -10 %). The lysis buffer to culture volume ratio was 1:10. The solution was sonicated having 30 sec on/off cycle for 6 such cycles. Centrifuged at 12,000 rpm for 30 minutes at 4°C. The protein was in the supernatant fraction. The supernatant was then subjected to Ni/NTA purification.

MAKING MONOCLONAL ANTIBODY AGAINST TNF ALPHA

To generate a monoclonal antibody TNF alpha protein was injected in BALB/c mice, by following the immunization schedule. Splenocytes were isolated and fused with Sp2O a mouse myeloma. After cell fusion, the hybrid cells which have myeloma plus IgG secreting characters were selected by Hypoxanthine-Aminopterin-Thymidine (HAT) selection media. Selected clones are expanded into culture flasks from 96 well plate, got 13 such clones which were screened for its reactivity by ELISA and Western blotting and functional screening by L929 and glucose uptake assay.

MAKING POLYCLONAL ANTIBODY AGAINST TNF ALPHA

New Zealand white rabbits of 4-6 weeks of ~ 2 kg were taken for the experiment. The following animal husbandry conditions were maintained: Temperature 20±3°C, Humidity 30-70 %, 12 hours light and 12 hours dark cycle, 12-15 air changes per hour. On day 0 pre-immune serum was collected (PIS) and the rabbits were immunized at sub cutaneous region with 300 µg/rabbit of TNF alpha protein. After 21 days of immunization rabbits were given 1st booster with 150 µg/rabbit of TNF alpha protein. 2nd booster again after 21 days with 300 µg/rabbit of TNF alpha protein was administered. Blood was collected on the 10th day after 2nd booster and the same schedule was followed for further boosters. This is Hyper-immune sera (HIS). Serum was separated from the blood for both PIS and HIS. Titer of both the rabbits was checked by in-direct ELISA. Based on the titer results 1 ml serum from 2nd rabbit was taken for Protein A purification. The purified antibody was checked for its purity by running it on SDS-PAGE. Thus, purified antibody was further conjugated to HRP and was used for sandwich ELISA.

SANDWICH ELISA PROTOCOL

Captured antibody was coated at 50 µl/well in sodium-bi-carbonate buffer (pH 9.4) on to the ELISA plates, incubated overnight at 4ºC. After overnight, washed with PBS and blocked with blocking buffer for 1 hour at room temperature. After 1 hour of blocking, the analyte was added which was prepared in blocking buffer, the plates were incubated for 1 hour at room temperature. After incubation the plates were washed three times with 1 X phosphate buffered saline with 0.05 % tween-20 (pH 7.2) followed by three times with 1 X phosphate buffered saline (pH 7.2) and detection antibody was loaded to the wells in blocking buffer. The plate was incubated for 1 hour at room temperature. After 1 hour incubation, the plate was washed three times with 1 X phosphate buffered saline with 0.05 % tween-20 (pH 7.2) followed by three times with 1 X phosphate buffered saline (pH 7.2). The developing solution 3,3',5,5'-Tetramethylbenzidine (TMB)-H_2O_2 was loaded to all the wells at 50 µl/well volume. The plate was incubated for 30 min in dark. The reaction was stopped by adding 25 µl/well of 2N H_2SO_4 to all the wells. The OD was taken at 450 nm. For all the next assays the same sandwich ELISA protocol was followed with the changes mentioned in the respective sections.

ESTABLISHING HIGH FAT DIET C57BL/6 MICE MODEL

C57BL/6 male mice with Age: 4-6 weeks old, Body weight: 15-21 g, Glucose values: 125-150 mg/dL were grouped 6 animals per group as:

Group 1 High Fat Diet - HFD with the following formulation [Vanaspati and Coconut oil (3:1 v/v) fed 3 ml/kg body weight per day along with 25% fructose in drinking water](Munshi *et al.*, 2014).

Group 2 High Fat Diet + Plant extract – HFD+PE with the following formulation [Vanaspati and Coconut oil (3:1 v/v) fed 3 ml/kg body weight per day along with 25 % fructose in drinking water] along with 300 mg/kg bodyweight of the methanolic Soxhlet extract of leaf.

Group 3 Normal diet – ND with normal rodent chow feed.

Blood was collected between 10 am to 12 pm, on day 0, week 4, 6, 8, 12 and 16 around 100 µl from which ~ 50 µl of serum was obtained. The weight of the animals and the blood glucose levels were recorded on the day of every blood collection. Serum TNF alpha, blood glucose was estimated at every time point.

PREPARATION OF METHANOLIC EXTRACT

- ✓ **Collection of plant material**

Seeds, bark and leaves from the plant are used in treatment of various diseases. However, with the seasonal limitation of availability, only leaf and bark were collected for this study from a village near Soraba taluk, Shivamogga district, Karnataka state in India, multiple times in the year 2018. Seasonal variation in secreted phytochemicals was ruled out by mixing samples collected throughout the year. The plant material was dried in shade and powdered in the grinder (Shahzadi *et al.*, 2017) before taking for further analysis.

- ✓ **Preparation of methanolic extract**

Polar solvents like methanol or ethanol can dissolve alkaloids, flavonoids, tannins, terpenoids, carbohydrates, steroids, terpenoids and proteins, which are the major components of interest in plants(Karthishwaran *et al.*, 2010). *H. antidysenterica* leaf and bark were subjected to extraction in methanol. Leaf and bark powder, 2 g each was extracted individually in Soxhlet apparatus in 200 ml of methanol. Extraction was carried out till the solvent in extraction chamber turned colorless. The semi-solid oleoresin was then concentrated by air drying and used for further analysis.

- ✓ **Qualitative and quantitative phytochemical estimation**

Qualitative assessment of fat, carbohydrates, protein and amino acids, alkaloids, glycosides, saponins, tannins, phenolic content, flavonoids, terpenoids and steroids were done according to the protocols by (Morsy, 2014; Tanvir *et al.*, 2017; Kunatsa *et al.*, 2020) briefly described below.

PHYTOCHEMICAL SCREENING TESTS

- ➢ **Test for total fat:** 1 g of leaf and bark with 200 ml of petroleum ether were placed in an extraction thimble separately for 2 hours. The solvent was evaporated, and total fat quantified by weight.

- **Test for carbohydrates:** Extracted leaf and bark were dissolved separately in 50 ml of water and filtered and the filtrate was subjected to Benedict's test:
- **Test for proteins and amino acids:** Leaf and bark extracts, 100 mg each were dissolved separately in 10 ml of distilled water and filtered through Whatman No.1 filter paper. The filtrate was used to perform Millon's test for detecting protein and amino acid content.
- **Test for alkaloids:** 50 mg of extract was treated with 2 ml of diluted HCl and was filtered. Mayer's, Wagner's, and Hager's tests were conducted to confirm alkaloid content:
- **Test for glycosides:** Bark and leaf extracts, 50 mg each, were hydrolyzed separately with concentrated HCl for 2 hours on a boiling water bath. Hydrolysate was filtered and subjected to ferric chloride test:
- **Test for saponins:** Saponins content in plant extract was detected by foam formation by continuously agitating with distilled water.
- **Test for phenolic compounds and tannins:** 50 mg of leaf and bark extracts were dissolved in distilled water and the mixture was treated with, 3 ml of 10 % lead acetate solution, bulky white precipitate indicates phenolic content in the extract.
- **Test for flavonoids:** Aqueous solution of leaf and bark extract were treated with 10 % NH_4OH. A yellow fluorescence indicates the presence of flavonoids.
- **Test for terpenoids and steroids:** Leaf and bark extracts, 2 mg each, were dissolved in 1 ml of chloroform with equal volume of concentrated H_2SO_4 which was added along sides of the tube. Appearance of red-brown ring at the junction of the two layers indicates the presence of terpenoids and the upper green layer indicates the presence of steroids.
- **Quantitative estimation of carbohydrates:** Leaf and bark extracts, 100 mg each was hydrolyzed with 2.5 N HCl then quantified by adding Anthrone reagent. The green to dark green color was read at 630 nm and plotted on standard curve of glucose to quantify carbohydrate (Morsy, 2014).
- **Quantitative estimation of proteins and amino acids:** Leaf and bark extracts, 500 mg was taken separately and ground well in 10 ml of the 0.2 M phosphate buffer, centrifuged at 10000 rpm for 10 minutes, 0.1 ml and 0.2 ml of the supernatant was taken for protein estimation after diluting to 1 ml with water by adding Folin-Ciocalteu reagent. Bovine serum albumin was used as standard (Morsy, 2014).
- **Quantitative estimation of alkaloids:** Leaf and bark extracts, 2 g was mixed separately with 50 ml each of 5 N HCl with continuous stirring followed by filtration. The filtrate was washed four times with 25 ml chloroform each time dried over anhydrous sodium sulphate and evaporated in hot air oven. The difference in the weight of the beaker gave the weight of total alkaloids present (Morsy, 2014).

- **Quantitative estimation of tannins:** Folin-Ciocalteu reagent (1:1 in distilled water), 0.5 ml, was added to 100 mg of leaf and bark extracts and treated with 2.5 ml of 20% solution of Na_2CO_3. The mixture was incubated for 40 minutes at ambient temperature. The absorbance was read at 725 nm in a plate reader using tannic acid as standards and methanol as control(Tanvir *et al.,* 2017).
- **Quantitative estimation of phenolic compounds:** Folin-Ciocalteu reagent (1:10 in distilled water), 0.5 ml, was added to 100 mg of leaf and bark extracts and treated with 4 ml of 7.5 % solution of Na_2CO_3. The mixture was incubated for 40 minutes at ambient temperature. The absorbance was read at 765 nm in a plate reader using gallic acid as standards and methanol as control(Morsy, 2014; Tanvir *et al.,* 2017).
- **Quantitative estimation of flavonoids:** Leaf and bark extracts, 100 mg was dissolved separately in 2 ml of distilled water followed by addition of 0.15 ml of 5 % sodium nitrite. This mixture was allowed to stand for 6 minutes, added 0.15 ml of 10 % aluminum trichloride and allowed to stand for another 6 minutes. To this mixture, 2 ml of 4 % sodium hydroxide solution was added followed by 3 ml of distilled water. After 15 minutes of incubation the pink color formed was measured at 510 nm using quercetin as standard and methanol as control(Morsy, 2014; Tanvir *et al.,* 2017).
- **Quantitative estimation of fat:** Leaf and bark extracts, 1 g was mixed separately with 200 ml of petroleum and placed in an extraction thimble for 2 hours. The solvent was evaporated, and total fat was quantified by weight(Almazan and Adeyeye, 1998).

DETERMINATION OF ANTIOXIDANT PROPERTY

1,1-Diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay is the most extensively used method to measure antioxidant potential of plant extracts. DPPH, a stable free radical, reacts with compounds that can donate a hydrogen atom(Aqil *et al.,* 2014; Priyanka *et al.,* 2017; Tanvir *et al.,* 2017). Various concentrations of analyte and ascorbic acid standards (1, 10, 25, 50, 75 and 100 µg/ml) were prepared from 2 mg/ml stock solution in methanol. This 1 ml of each leaf/bark and standards, was mixed with 3 ml of DPPH ($6x10^{-5}$M) and incubated in dark for 15 minutes at ambient temperature. Absorbance was measured at 517 nm using methanol and only DPPH solution as controls. Percentage of inhibition at each concentration was calculated. Concentration versus % inhibition data was fitted to a 4-parameter logistic equation using GraphPad Prism 5 software and IC_{50} value with 95 % confidence interval (CI) were obtained from the fit.

CULTURE OF MONOCLONAL ANTIBODY AND PURIFICATION

The 13 monoclonal hybridoma clones obtained were cultured in IMDM media with 10% FBS. Passaged every 3 days, the spent media was collected from each passage which has the secreted

antibody in it. The antibody was purified on Protein A beads. The purified antibodies were screened for its binding activity by ELISA and Western blotting and neutralizing activity by L929 and glucose uptake assay.

SCREENING OF MONOCLONAL ANTIBODIES AGAINST TNF

Western blot analysis was performed to screen 13 monoclonal antibodies against TNF alpha on whole cell extracts (20 µg lysate) of TNF alpha expressing *E. coli*. The blots were probed with culture supernatant of 13 monoclonal antibodies, overnight at 4ºC on after blocking for 1 hour with 5% skimmed milk followed by using Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP conjugate as secondary antibody. The blots were developed using ECL Substrate Reagent Kit in LAS4000 imager.

L929 ASSAY TO SCREEN THE NEUTRALIZING ANTIBODY ON RECOMBINANT TNF ALPHA PROTEIN

Seeded 30 thousand cell/well of L929 cells in 96 well cell culture plate. Incubated overnight (O/N) at 37ºC with 5% carbon dioxide. Next day cells were treated with 100 ng/ml of recombinant TNF alpha protein along with 200, 100 and 50 ng/ml of H4D6, H4A3, 4H11 and H4B1 antibodies individually in the presence of 5 µg/ml of Daunorubicin for 16 hours at 37ºC with 5% carbon dioxide. Recombinant TNF alpha protein alone at 100 ng/ml and 5 µg/ml of Daunorubicin alone were taken as control (Shiau *et al.*, 2017). After 24 and 48 hour of treatment the cells were taken for Alamar blue staining, and the cytotoxicity was calculated.

HIGH GLUCOSE INDUCED INSULIN RESISTANCE

Seeded HepG2, 30,000 cells/well in 96 well cell culture plate was grown in 5.5 mM of glucose (100 mg/dl the physiological normo-glucose), for 48 hrs. Cells were serum starved for 20 hours and stimulated with insulin in serum free medium containing with 0, 20, 200, 2000 and 5000 nM for 1, 2, 4 and 6 hours at 37°C(Lo *et al.*, 2013). The supernatant was collected and taken for glucose estimation by glucose estimation kit from Erba. To induce insulin resistance, HepG2 cells were grown for 48 hours in different concentrations of glucose i.e., 5.5, 10, 12, 25, 40 and 50 mM. Cells were serum starved for 20 hours followed by challenging with 0 and 2000 nM insulin in serum free medium with respective concentrations of glucose for 2 hours at 37°C. Glucose was estimated in the supernatant by glucose estimation kit from Erba.

SCREENING THE MONOCLONAL ANTIBODIES ON GLUCOSE UPTAKE ASSAY

Seeded 30K HepG2 cells/well in 96 well plate for 48 hours in 50 mM glucose. After 48 hours the cells were serum starved for 20 hours. Treatment was done in serum free medium with H4D6, H4A3, 4H11, H4B1 and polyclonal antibodies at 5, 2.5 and 1.25 nM along with 2000 nM of insulin and 2.5 nM of TNF alpha was mixed and incubated for 1 hour at 37°C. After incubation

the cells were treated for 2 hours at 37°C with the above mixture. Only TNF alpha at 2.5 nM, only insulin at 2000 nM and TNF alpha at 2.5 nM plus 2000 nM of insulin were taken as control. After the incubation, media were collected and taken for glucose estimation by the glucose estimation kit from Erba.

mRNA SEQUENCING OF INSULIN RESISTANT Hepg2

Cells after appropriate treatment were stored in -80°C with TRIzol overnight. Next day RNA was extracted by chloroform precipitation method manually. RNA was quantified using Qubit RNA Assay BR. Purity was checked using QIAxpert and RNA integrity was assessed on TapeStation using RNA ScreenTapes. A modified Illumina TruSeq RNA Sample Preparation V2 protocol was used for making RNA sequencing libraries. The adapter ligated products were then purified and enriched using the following conditions: initial denaturation at 98°C for 30 sec; 15 cycles of 98°C for 10sec, 60°C for 30sec, 72°C for 30sec; final extension of 72°C for 5mins. PCR products were then purified and checked for fragment size distribution on TapeStation D1000 DNA tapes. The obtained libraries quantified and pooled, followed by diluting to final optimal loading concentration before cluster amplification on Illumina flow cell. Once the cluster generation is completed, the cluster flow cell was loaded on Illumina HiSeq X instrument to generate 30M, 150bp paired end reads.

ANALYSIS OF EMERGING PATHWAYS

Sequenced reads were pre-processed using the Fastp tool(S. Chen *et al.,* 2018). Transcript abundances from the pre-processed reads were quantified using Salmon(Patro *et al.,* 2017) alignment-free quantifier, which were summed up to gene-level quantification using the tximport tool(Soneson *et al.,* 2016). Differential gene expression between the treatment and control samples were computed using the edgeR Bioconductor package(Robinson *et al.,* 2009). ClusterProfiler(Yu *et al.,* 2012), Bioconductor package, was used to enumerate over-represented GO biological processes and KEGG metabolic pathways from differentially expressed genes. Differential gene expression and pathway analysis using the above packages were carried out on R statistical computing platform(R Development Core Team 3.0.1., 2013).

DOSE DEPENDENT REVERSAL OF INSULIN RESISTANCE BY ANTI-TNF ALPHA CLONE 4H11

Insulin resistant cells (50 mM glucose) were stimulated with 2000 nM Insulin in serum free medium with different concentrations (0.125, 0.083, 0.056, 0.037 and 0.025 nM) of anti-TNF alpha antibody, clone 4H11 for 2 hours at 37°C(Chen *et al.,* 2018). After incubation, media was collected and glucose was estimated by glucose estimation kit from Erba.

HIGH GLUCOSE INDUCED OXIDATIVE STRESS

HepG2 cells both grown in 5.5 mM and 50 mM glucose were stimulated with 2000 nM insulin for 2 hours at 37°C. After stimulation cells were stained with 10 μM of 2′,7′-Dichlorofluorescin diacetate (DCFH-DA; Sinisgalli *et al.,* 2020) the single cell suspension was loaded on to BD FACSCanto II and captured at λex 485 nm and λem 515–540 nm(Sinisgalli *et al.,* 2020).

HIGH GLUCOSE INDUCED LIPOGENESIS

HepG2 cells grown in 5.5 mM and 50 mM glucose were stimulated with 2000 nM insulin for 2 hours at 37°C. After treatment cells were stained with Oil-O-Red. Cells were kept covered with distilled water at all times and while imaging under microscope (Yao *et al.,* 2011; Kraus *et al.,* 2016). Oil-O-Red stain was extracted with 100% isopropanol for 5 min with gentle rocking and reading was measured with OD at 492nm.

EXTRACTION OF LEAF AND BARK IN DIFFERENT SOLVENTS

The extraction of bark and leaf was carried out manually at room temperature(Upadhayay, 2016) and by Soxhlet(Verran *et al.,* 2014) in the following solvents: Water, chloroform, hexane, 70 % ethanol(Verran *et al.,* 2014; Sharma *et al.,* 2015), acetone, ethyl-acetate, methanol(Karthishwaran *et al.,* 2010), and petroleum ether. This range of solvents from polar to non-polar dissolve almost all the phytochemicals present in the plants for example alkaloids, flavonoids, tannins, terpenoids, carbohydrates, steroids, terpenoids and proteins(Karthishwaran *et al.,* 2010).

CYTOTOXICITY OF THE EXTRACTS ON HepG2

To screen the extracts on HepG2 for their cytotoxic concentration, cells were seeded 30K/well in 96 well cell culture plate for 24 hours. After 24 hours cells were treated with leaf and bark extracts individually at 10, 5 and 2.5 mg/ml concentration for another 24 hours. After the treatment Alamar blue was added and reading at 570 and 600 was taken after 4 hours. After Alamar blue reading, the cells in the 96 well plate were washed with distilled water and stained with crystal violet for 20 min at RT(Shiau *et al.,* 2017). After incubation, the stain was washed once with distilled water, images of the cells were captured after air drying the plate.

EFFECT OF *H. ANTIDYSENTERICA* ON INSULIN RESISTANCE

Insulin resistant HepG2 cells were treated with sub cytotoxic concentrations of the plant extracts with 2000 nM insulin for 2 hours at 37°C. Cells with no treatment and with respective solvents was kept as controls. The supernatant was collected and taken for glucose estimation by the kit from Erba.

H. ANTIDYSENTERICA AS AN ANTIOXIDANT

The insulin resistant HepG2 cells were treated with sub cytotoxic concentrations of the plant extracts with 2000 nM insulin for 2 hours at 37°C. Cells with no treatment and with respective solvents was kept as controls. After treatment cells were stained with 10 μM of 2′,7′ Dichlorofluorescin diacetate.

EFFECT OF *H. ANTIDYSENTERICA* ON LIPOGENESIS

The insulin resistant HepG2 cells were treated with sub cytotoxic concentrations of the plant extracts with 2000 nM insulin for 2 hours at 37°C. Cells with no treatment and with respective solvents were kept as controls. After treatment cells were stained with Oil-O-Red as described in the earlier section.

STATISTICAL ANALYSIS

All the tests were performed in triplicates (n=3). Each value is expressed as mean ± standard deviation. Statistical significance was assessed using two-way ANOVA (* $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$) as compared to standard group using Graph Pad Prism 5 software.

CHAPTER 4. RESULTS AND DISCUSSION

TNF ALPHA PROTEIN EXPRESSION AND PURIFICATION

TNF alpha protein was cloned between HindIII and XhoI restriction sites into pET-28a(+) vector with the forward and reverse primers as follows:

Forward primer - CCCAAGCTTGCCATGCTCAGATCATCTTCTC

Reverse Primer - CCGCTCGAGTGACAGAGCAATGACTCC

The protein was expressed, purified on NiNTA column and found to be running at ~ 26 kDa on an SDS-PAGE and confirmed by Western blot probed with Anti-HIS antibody. As this protein is having 6HIS tag, can be detected with Anti-HIS antibody.

TNF ALPHA PROTEIN INDUCES CYTOTOXICITY ON L929 ASSAY

The activity of TNF alpha when checked on L929 assay, showed a dose dependent cytotoxicity, wherein % cytotoxicity is plotted vs different concentrations of TNF alpha from 1000 to 0.0001 ng/ml.

TNF ALPHA PROTEIN IMPAIRS THE GLUCOSE UPTAKE

HepG2 cells were treated with TNF alpha protein with different concentrations (100, 50, 25 and 12.5 ng/ml) and glucose in the media was measured at 1, 2, 4 and 6 hours after the treatment. The

glucose left in the media was quantified which was inversely proportional to the glucose taken up by the cells. The difference in glucose uptake started showing up at 2 hours compared to no treatment. The presence of 100 and 50 ng/ml of TNF alpha prevented glucose uptake even after insulin stimulation.

ESTABLISHMENT OF SANDWICH ELISA FROM POLYCLONAL ANTIBODY

Titer was checked after 2nd booster and found that rabbit 2 showed better titer when compared with rabbit 1 so the serum from rabbit 2 was taken for purification on Protein A beads. Total yield was 16.94 mg of antibody. There were 2 bands corresponding to heavy ~ 55 kDa and light chains ~25 kDa of the antibody observed when run on SDS-PAGE. Antibody, 0.5 mg was taken for conjugation with 0.5 mg of HRP. After conjugation the volume was 0.1 ml. For this, 0.1 ml of stabilization was added and stored at -20°C.

- **Establishing the standard curve in assay matrix**

The TNF alpha standard curve was established from 3 to 0.0012 µg/ml of TNF alpha concentrations. The percentage analytical recovery (% AR) was calculated in the GraphPad Prizm software with the help of the formula: y = d + (a - d)/ (1 + (x / c)^b). Among the five Quality Check (QC) points of ULOQ-230.77 ng/well, HQC-136.55 ng/well, MQC-80.80 ng/well, LQC-47.81 ng/well and LLOQ-28.29 ng/well tested in the assay only ULOQ did not pass the criteria of % AR, which should be in the range between 80 to 120 %.

The three standard curves, each performed with triplicate readings showed that the assay is reproducible with in the validation range of 3 to 0.0012 µg/ml. The established ELISA assay was validated and was ready to be used for sample analysis.

ESTABLISHMENT OF DIABETIC MICE MODEL

- **Establishing high fat diet induced diabetic mice**

The high fat high sugar diet induced *in-vivo* C57BL/6 mice model was built, in which there was no significant difference in body weight and glucose values, but the TNF alpha levels were higher in HFD group when compared to ND group, where 29.76 ± 5.1 mg/dl was the TNF alpha levels in HFD group and 24.86 ± 1.63 mg/dl was in ND group.

TO ESTABLISH *IN-VITRO* DIABETIC MODEL IN HepG2 CELLS

- **High glucose induced IR**

HepG2 cells grown at 5.5 mM were stimulated with various concentrations of insulin for varied duration. It was found that at 2 hours with 2000 nM of insulin the HepG2 cells started showing glucose uptake. With this concentration of insulin and time of stimulation we opted to the next set of experiments. When cells were stimulated with different concentrations of insulin over time,

it was found that glucose uptake started happening at 2 hours with 2000 nM of insulin. So, this time point, and insulin concentration was fixed for further experiments. HepG2 cells were grown in different concentrations of Glucose starting from 5.5 mM till 50 mM and cells were stimulated with 2000 nM Insulin for 2 hours and glucose in the media was measured. It was found that Insulin resistance peaked at 50 mM glucose, starting from 40 mM of glucose in growth media.

- **Increased oxidation and lipogenesis with induced insulin resistance:**

DCFH is a nonfluorescent dye but switches to highly fluorescent DCF when oxidized by intracellular ROS or other peroxides(Rastogi *et al.*, 2010). ROS production in insulin resistant HepG2 cells was measured by the percentage of DCFH-DA (highly fluorescent) stained cells which was higher in 50 mM (86.67+/-2.4 %) when compared to 5.5 mM (76.6+/-3.4 %) cells.
Oil-O-Red stains neutral lipids (triglycerides and diacylglycerols), in addition to cholesterol esters with a maximum absorption at 518 nm. Numbers and sizes of the lipid droplets increased upon glucose shock. Extracted Oil-O-Red stain with 100 % Isopropanol supports the results with increased OD at 50 mM glucose cells (Khorzoughi *et al.,* 2019).

- **RNA isolation and sequencing**

RNA with distinct three bands and RIN value greater than 7 was taken for mRNA sequencing. The experiment was done in triplicates and the QC was done by running the RNA on 1% agarose gel. RNA was quantified using Qubit RNA Assay BR. Purity was checked using QIAxpert and integrity was assessed on TapeStation using RNA ScreenTapes.

- **Analysis of emerging pathways**

Differential gene expression analysis revealed several genes were upregulated and downregulated which indicated (i) upregulated TNFα signaling and loss of control in (ii) oxidative pathway and (iii) lipid metabolism pathways. Involvement of oxidative stress and impaired lipid metabolism pathways were validated by functional assays. It was found that IL-17, MAPK and TNF signalling pathways were upregulated in insulin resistant cells. These pathways are interconnected, and TNF alpha is the central molecule in it. Insulin receptor (INSR) exists in two isoforms A and B with varied affinity towards insulin. INSR-A with 12 amino acids less in the binding pocket cannot bind to insulin as tightly as INSR-B does(Malakar *et al.,* 2016). The ratio of INSR-A to -B mRNA is significantly increased in HepG2 with 50 mM glucose, explaining the mechanism of insulin resistance at the receptor level.

TO MAKE NEUTRALIZING ANTI-TNF ALPHA MONOCLONAL ANTIBODY

- **Screening the Monoclonal antibodies on ELISA and Western Blot:**

When screened on ELISA, all the 13 antibodies reacted on western blot only H4D6, H4A3, H4B1

reacted lighting up a 26 kDa protein band corresponding to TNF alpha protein. 4H11 and THP gave a very faint band at 26 kDa. Finally based on the yield upon Protein A purification, H4D6, H4A3, 4H11 and H4B1 were taken forward. These selected antibodies were screened for its neutralizing capacity.

- **Screening the Monoclonal antibodies on L929 assay:**

Antibodies 4H11, H4B1 and H4A3 were able to neutralize TNF-alpha induced cytotoxicity on L929 cells. 4H11 was the best neutralizing antibody with > 60 % reduction in the % cytotoxicity. Percentage cytotoxicity was calculated by taking only TNF alpha treated cells as 100%.

- **Screening the antibodies on glucose uptake assay:**

When screened 4H11, H4B1, H4D6 and H4A3 for their effect on glucose uptake, all the antibodies could increase the glucose uptake. 4H11 was the best antibody in reversing the TNF alpha induced insulin resistance and enhancing the glucose uptake by the cells. For further experiments 4H11 will be selected for the study of the insulin signaling mechanisms.

- **4H11 on glucose uptake:**

Treatment of insulin resistant cells with anti-TNFα monoclonal antibody reversed the disease condition. There was a dose dependent increase in glucose uptake observed when treated with different concentrations of 4H11.

- **Emerging pathway analysis:**

When sequencing analysis of 50 mM glucose treated cells with and without treatment with 4H11 was done it was found that IL-17 pathway, which was upregulated in the insulin resistant model, was downregulated upon treatment with anti-TNF antibody 4H11. The INSR-A/B ratio was also low upon treatment with 4H11 compaired to the control 50 mM HepG2. Differential gene expression studies with the treatment on anti-TNF alpha antibody 4H11 showed that, NBEAL1, AKT3, ONECUT1 and NOTCH2 genes were upregulated and other genes like PRR3, RGL2, FOS, FOSB and C4A were downregulated.

***H. ANTIDYSENTERICA* EXTRACTION AND PHYTOCHEMICAL ANALYSIS**

- **Meta-analysis on H. antidysenterica:**

Longitudinal meta-analysis of the data available on blood glucose levels showed that leaf and seed extracts of *H. antidysenterica* did reduce the blood glucose.

Meta-Regression and Subgroup Analysis showed extract from seeds have enhanced glucose-lowering ability (SMC 16.49, 95% CI: 7.99, 25.90) compared to that of leaf (SMC 3.42, 95% CI: 0.55, 6.29). In meta-analysis of other markers: Both seed and leaf extract and standard drug

reduced total cholesterol and triglyceride. Seed and leaf extract had marginally significant effect on total serum protein.

- **Extraction of leaf and bark in Soxhlet and by manual method:**

Ethanol was the highest yielding solvent, followed by methanol and water. Petroleum ether and Hexane were the least yielding solvents. Yield was higher in all the Soxhlet extracts except for bark in 70% ethanol and leaf in water. There was no difference in the yield between Soxhlet and manual methods when bark was extracted in methanol.

- **Cytotoxicity of the extracts on HepG2:**

It was found that leaf and bark when extracted in hexane were the most cytotoxic followed by 70% ethanol to be moderately cytotoxic and acetone was least. Among the bark and leaf across solvents, bark was more cytotoxic. Among the Soxhlet and manual, samples extracted in Soxhlet was more cytotoxic than in manual. Sub cytotoxic concentration were taken for further assays.
The Soxhlet extracted methanolic extract of leaf and bark were subjected to phytochemical analysis and checked for its antioxidant capacity.

- **Qualitative phytochemical estimation from methanolic extracts:**

Preliminary qualitative screening of the phytochemicals in *H. antidysenterica* revealed the presence of flavonoids, tannins, alkaloids, glycosides, saponins, carbohydrates, proteins and amino acids, total fat, total phenol content, terpenoids, steroids in Soxhlet extracts of leaf and bark with methanol.

- **Quantitative estimation for selected phytochemicals from methanolic extracts:**

Quantitative estimation was performed for selected phytochemicals, found quantifiable presence of flavonoids, tannins, alkaloids, proteins and amino acids, total fat and total phenol content and expressed as respective standard equivalents per g of the extracts. Carbohydrate content present in the leaf and bark extracts as it was below the limit of quantification. The extraction results indicated that leaves have higher estimation of all phytocompounds, except total fat which was higher in bark.

- **Antioxidant property:**

A 4-parameter logistic equation using GraphPad Prism 5 software was used to calculate IC_{50} value, 95 % confidence interval was obtained for both leaf and bark from the fit. DPPH scavenging activity of methanolic extracts of bark and leaf had IC_{50} values of 9.27 µg/mL; 95% CI: 7.41 - 11.60 and 6.55 µg/mL; 5.23 - 8.21 respectively with IC_{50} of ascorbic acid was 1.38 µg/mL; 1.26 - 1.52.

- **Quantitative estimation phytochemicals from water extracts:**

As water extracts were best in increasing glucose uptake, phytochemicals present in them were quantified and found high levels of carbohydrates and tannins.

***H. ANTIDYSENTERICA* ON *IN-VITRO* AND *IN-VIVO* MODEL**

- ***H. Antidysenterica* extract in blocking of TNF-alpha along with glucose in diabetes induced animals**

Leaf extract was applied for the treatment in *in-vivo* HFD induced diabetic animal model. There was no difference in the body weight between HFD and HFD+PE group up to 16 weeks of observation. The glucose values in the HFD+PE group started showing a decline after 8 weeks of treatment, which followed up to 16^{th} week. The maximum glucose dip in the HFD+PE group was observed at 12^{th} week up to 29.5 ± 3 mg/dl from 31.0 ± 3.6 mg/dl in HFD group. The TNF alpha levels were not different till 8 weeks of treatment. Only on 12^{th} week there was decrease in the TNF alpha levels observed in HFD+PE group up to 22.0 ± 5.0 µg/ml from 29.76 ± 12.4 µg/ml in the HFD.

- **Effect of *H. antidysenterica* on insulin resistance:**

Water extracts of bark and leaf when extracted in either by Soxhlet or manual method was best in dose dependent reversal of insulin resistance. With four decreasing concentrations of the extracts: bark when extracted by Soxhlet showed 145.97, 126.00, 122.93 and 113.66%, when extracted manually 142.67, 124.43, 122.36 and 109.05%, leaf when extracted by Soxhlet showed 144.07, 121.99, 115.17 and 111.34%, when extracted manually 141.66, 123.58, 112.25 and 107.60 % of glucose being taken up by the insulin resistant HepG2. It was found that Soxhlet extraction gave better yield and also reversal of insulin resistance was more in the leaf and bark when extracted by Soxhlet, so samples extracted in Soxhlet were taken for anti-oxidant and anti-lipid screening.

- ***H. antidysenterica* as an antioxidant:**

The results showed a dose dependent decrease in ROS production when treated with Soxhlet extracts of bark and leaf extracted in 70% ethanol, bark extracted in acetone and leaf extracted in water. Bark in acetone had the lowest ROS production with only 24.73 % of DCFH-DA stained cells as shown in figure 45 and 46. Manually extracted bark was also checked for its capacity to reduce the ROS production, it had no effect on ROS production.

- **Effect of *H. antidysenterica* on lipid deposition:**

The results showed that bark extracted in soxhlet with water, acetone, petroleum ether, chloroform and methanol and leaf extracted in hexane showed a dose dependent decrease in lipid

deposition. Among them bark extracted in acetone was the best so checked the manually extracted bark also for its capacity to reduce the lipid deposition. The manually extracted bark in acetone could also reduce lipid deposition.

The thesis deals with each of the above topics in detail.

CHAPTER 5 - SUMMARY AND CONCLUSION

SUMMARY

This thesis deals mainly with the preliminary qualitative, quantitative phytochemical screening, antioxidant activity of extracts of *Holarrhena antidysenterica* leaf and bark. Furthermore, development of HepG2 insulin resistant cell line an *in vitro* model for diabetes with impaired oxidative and lipogenesis pathways. Developed an *in vivo* diabetic model in mice. Testing of all the extracts and Anti-TNF alpha antibody on these *in vitro* and *in vivo* models. Also studied the emerging molecular pathway and IR-A/IR-B ratio in HepG2 insulin resistant cell line an *in-\vitro* model. All these have been described in different chapters and the summary and conclusion for each chapter have been described below:

Chapter I

This chapter initially deals with the introductory details of cancer and its prevalence, nanotechnology, and their applications. The need for choosing the medicinal plant and their advantages were explained in detail. Furthermore, the general information of ZnO, and CuO NPs and their application are given in detail.

Chapter II

This section gives the literature relevant to the present study. A summary on the different parts *H. antidysenterica* used in the treatment of diabetes. Current medication for diabetes and how TNF alpha can be targeted to reduce the complications of diabetes. The literature search provides the background and guidance for the scope of the present investigation.

Chapter III

This chapter briefly covers the materials required and instruments used for the study. Initially chapter deals with collection of plants, preparation of extract, phytochemical screening, quantification, and antioxidant activity. Furthermore, the techniques for developing polyclonal and monoclonal anti-TNF alpha antibody, ELISA development to detect TNF alpha from mice serum, establishment of *in-vitro* and *in-vivo* models of diabetes. Detailed protocol used for testing the plant extracts and anti-TNF alpha antibody on these *in-vitro* and *in-vivo* models to reduce diabetes. At the end, the chapter dealswith the assay procedures of estimating ROS and lipid deposition in HepG2 cells.

Chapter IV

This chapter deals with results and discussions of the entire study. Starting from preliminary physicochemical screening, quantification, and antioxidant activity. Briefly the study revealed that preliminary phytochemical analysis revealed the presence of flavonoids, tannins, alkaloids, glycosides, saponins, carbohydrates, proteins and amino acids, total fat, total phenol content, terpenoids, steroids. All the phytochemicals present could be quantified, only carbohydrate content was too low to be quantified.

Furthermore, application of all 32 extracts onto insulin resistant HepG2 cells, where increased glucose uptake was observed upon treatment. The polyclonal antibody made was used to develop sandwich ELISA platform. This ELISA was used to check the TNF alpha levels in the high fat diet *in vivo* diabetic model. An *in vitro* diabetic model in HepG2 cell lines with increased oxidative stress and lipogenesis was successfully established. Successfully neutralized TNF alpha and reverse the high glucose induced insulin resistance in HepG2. Extracted bark and leaf of *H. antidysenterica* in different solvents and screened for its anti-diabetic activity in *in vitro* and *in vivo* models.

Rabbit Anti-TNF alpha polyclonal antibody was generated, got a yield of 16.94 mg from 1 ml of the sera. This antibody was conjugated to HRP and used as a detection for the sandwich ELISA where un-conjugated antibody was used as a capture. The ELISA was sensitive enough to detect TNF alpha till 0.001 µg/ml. This ELISA was used to estimate TNF alpha levels in mice serum from high fat diet induced diabetic model. TNF alpha levels were higher in HFD group on week 12 with 29.76 ± 12.49 mg/dl compared to ND with 24.85 ± 3.9 mg/dl. There was no significant difference observed in body weight and glucose values between the HFD and ND groups. *H. antidysenterica* revealed the presence of flavonoids, tannins, alkaloids, glycosides, saponins, carbohydrates, proteins and amino acids, total fat, total phenol content, terpenoids, steroids in Soxhlet extracts of leaf and bark with methanol. DPPH scavenging activity of methanolic extracts of leaf and bark as measured in this study have IC_{50} values 10.15 µg/mL (95% CI: 9.89 – 10.43) and 8.56 µg/mL (95% CI: 8.03 – 9.13) respectively. The maximum glucose dip in the HFD+PE group was observed at 12^{th} week up to 29.5 ± 3 mg/dl from 31.0 ± 3.6 mg/dl in HFD group with also decrease in the TNF alpha levels observed in HFD+PE group up to 22.0 ± 5.0 µg/ml from 29.76 ± 12.4 µg/ml in the HFD.

When screened the monoclonal anti-TNF alpha antibody for its neutralization effect, it was found that 4H11, H4B1 and H4A3 were able to neutralize TNF-alpha protein induced cytotoxicity on L929 cells. 4H11 was the best antibody in reversing the TNF alpha induced insulin resistance and enhancing the glucose uptake by the cells.

When HepG2 cells were grown in different glucose concentrations, insulin resistance was shown to be peaked at 50 mM, starting from 40 mM of glucose in growth media. The ratio of INSR-A to -B mRNA is significantly increased in HepG2 with 50 mM glucose along with IL-17 and TNF alpha pathways being up regulated. Treatment of insulin resistant cells with anti-TNF alpha monoclonal antibody 4H11 reversed the disease condition by increasing the glucose uptake. The INSR-A/B ratio was lowered upon treatment with 4H11 compared to the control. With four decreasing concentrations of the extracts, showed dose dependent glucose being taken up by the insulin resistant HepG2. ROS production in insulin resistant HepG2 cells was measured by the percentage of DCFH-DA-stained cells which was higher in 50 mM (86.67+/-2.4 %) when compared to 5.5 mM (76.6+/-3.4 %) cells. After treatment with the extracts with no treatment as a control. The results showed a dose dependent decrease in ROS production when treated with Soxhlet extracts of bark and leaf extracted in 70% ethanol, bark extracted in acetone and leaf extracted in water. Bark in acetone had the lowest ROS production with only 24.73 % of DCFH-DA-stained cells. Numbers and sizes of the lipid droplets increased upon glucose shock. The results show that bark extracted in water, acetone, petroleum ether, chloroform and methanol and leaf extracted in hexane showed a dose dependent decrease in lipid deposition.

CONCLUSION

In conclusion, we could successfully establish high glucose induced insulin resistance, oxidative stress, and lipid deposition in HepG2 cells. Also, we could show that high glucose induces ROS production which leads to TNF alpha mediated inflammation and increased insulin receptor splicing leading to diminished binding to insulin receptor B which leads to insulin resistance. This study is novel as it covers three pathways of diabetes in a single *in vitro* model unlike other studies involving HepG2. *Holarrhena antidysenterica* is a potential antidiabetic drug with its action on insulin resistance, oxidation, and lipid deposition pathways. *H. antidysenterica* also reduced TNF alpha and blood glucose levels in high fat diet induced *in vivo* models. Anti-TNF alpha neutralizing antibody could reverse the high glucose induced insulin resistance in HepG2.

REFERENCES

Almazan, A.M., Adeyeye, S.O., 1998. Fat and fatty acid concentrations in some green vegetables. J. Food Compos. Anal. 11, 375–380. https://doi.org/10.1006/jfca.1998.0596

Aqil, F., Munagala, R., Jeyabalan, J., Joshi, T., Gupta, R.C., Singh, I.P., 2014. The Indian Blackberry (Jamun), Antioxidant Capacity, and Cancer Protection, Cancer: Oxidative Stress and Dietary Antioxidants. Elsevier. https://doi.org/10.1016/B978-0-12-405205-5.00010-6

Chen, F.C., Shen, K.P., Chen, J.B., Lin, H.L., Hao, C.L., Yen, H.W., Shaw, S.Y., 2018. PGBR extract ameliorates TNF-α induced insulin resistance in hepatocytes. Kaohsiung J. Med. Sci. 34, 14–21. https://doi.org/10.1016/j.kjms.2017.08.009

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. Fastp: An ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34, i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Dandona, P., Aljada, A., Chaudhuri, A., Mohanty, P., Garg, R., 2005. Metabolic syndrome: A comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation. https://doi.org/10.1161/01.CIR.0000158483.13093.9D

Emanuelli, B., Peraldi, P., Filloux, C., Chavey, C., Freidinger, K., Hilton, D.J., Hotamisligil, G.S., Van Obberghen, E., 2001. SOCS-3 Inhibits Insulin Signaling and Is Up-regulated in Response to Tumor Necrosis Factor-α in the Adipose Tissue of Obese Mice. J. Biol. Chem. 276, 47944–47949. https://doi.org/10.1074/jbc.M104602200

Forsythe, L.K., Wallace, J.M.W., Livingstone, M.B.E., 2008. Obesity and inflammation: The effects of weight loss. Nutr. Res. Rev. 21, 117–133. https://doi.org/10.1017/S0954422408138732

Giacco, F., Brownlee, M., 2010. Oxidative stress and diabetic complications. Circ. Res. https://doi.org/10.1161/CIRCRESAHA.110.223545

Guilherme et la., 2008. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol 9, 367–377. https://doi.org/10.1038/jid.2014.371

Jamadagni, P.S., Pawar, S.D., Jamadagni, S.B., Chougule, S., Gaidhani, S.N., Murthy, S.N., 2017. Review of Holarrhena antidysenterica (L.) Wall. ex A. DC.: Pharmacognostic, pharmacological, and toxicological perspective. Pharmacogn. Rev. https://doi.org/10.4103/phrev.phrev_31_16

Karthishwaran, K., Mirunalini, S., Dhamodharan, G., Krishnaveni, M., Arulmozhi, V., 2010. Phytochemical investigation of methanolic extract of the leaves of pergularia daemia. J. Biol. Sci. https://doi.org/10.3923/jbs.2010.242.246

Khan, W., Subhan, S., Shams, D.F., Afridi, S.G., Ullah, R., Shahat, A.A., Alqahtani, A.S., 2019. Antioxidant Potential, Phytochemicals Composition, and Metal Contents of Datura alba. Biomed Res Int. 2019, 1–8. https://doi.org/10.1155/2019/2403718

Khorzoughi, R.B., Namvarjah, F., Teimouri, M., Hosseini, H., Meshkani, R., 2019. In-vitro synergistic effect of metformin and berberine on high glucose-induced lipogenesis. Iran. J. Pharm. Res. 18, 1921–1930. https://doi.org/10.22037/ijpr.2019.15085.12867

Kraus, N.A., Ehebauer, F., Zapp, B., Rudolphi, B., Kraus, B.J., Kraus, D., 2016. Quantitative assessment of adipocyte differentiation in cell culture. Adipocyte 5, 351–358. https://doi.org/10.1080/21623945.2016.1240137

Kunatsa, Y., Chidewe, C., Zvidzai, C.J., 2020. Phytochemical and anti-nutrient composite from selected marginalized Zimbabwean edible insects and vegetables. J. Agric. Food Res. 2, 100027. https://doi.org/10.1016/j.jafr.2020.100027

Kuryłowicz, A., Kózniewski, K., 2020. Anti-inflammatory strategies targeting metaflammation in type 2 diabetes. Molecules 25, 1–32. https://doi.org/10.3390/molecules25092224

Liu, L., Zhang, X., Chen, F., Hu, J., Zeng, B., 2017. The establishment of insulin resistance model. BIO Web Conf. 8, 1–7. https://doi.org/10.1051/bioconf/20170803005

Lo, K.A., Labadorf, A., Kennedy, N.J., Han, M.S., Yap, Y.S., Matthews, B., Xin, X., Sun, L., Davis, R.J., Lodish, H.F., Fraenkel, E., 2013. Analysis of In Vitro Insulin-Resistance Models and Their Physiological Relevance to InVivo Diet-Induced Adipose Insulin Resistance. Cell Rep. 5, 259–270. https://doi.org/10.1016/j.celrep.2013.08.039

Malakar, P., Chartarifsky, L., Hija, A., Leibowitz, G., Glaser, B., Dor, Y., Karni, R., 2016. Insulin receptor alternative splicing is regulated by insulin signaling and modulates beta cell survival. Sci. Rep. 6, 1–14. https://doi.org/10.1038/srep31222

Morsy, N., 2014. Phytochemical analysis of biologically active constituents of medicinal plants. Main Gr. Chem. 13, 7–21. https://doi.org/10.3233/MGC-130117

Mrinal and Navjeet Singh, 2018. A Review on Pharmacological Aspects of Holarrhena antidysenterica. Sch. Acad. J. Pharm. 7(12), 488–492. https://doi.org/10.21276/sajp.2018.7.12.5

Muhammad Sajid Hamid Akash, Kanwal Rehman, A.L., 2017. Tumor Necrosis Factor-Alpha Role in Development of Insulin Resistance and Pathogenesis of Type 2 Diabetes

Mellitus.pdf. J Cell Biochem. 119, 105–110. https://doi.org/10.1002/jcb.26174

Munshi, R.P., Joshi, S.G., Rane, B.N., 2014. Development of an experimental diet model in rats to study hyperlipidemia and insulin resistance, markers for coronary heart disease. Indian J. Pharmacol. 46, 270–276. https://doi.org/10.4103/0253-7613.132156

Parikh, N.H., Parikh, P.K., Kothari, C., 2014. Indigenous plant medicines for health care : treatment of Dia- betes mellitus and hyperlipidemia. Chin. J. Nat. Med. 12, 335–344. https://doi.org/doi: 10.1016/S1875-5364(14)60041-8

Patro, R. 2010-edgeR a bioconductor package. pd., Duggal, G., Love, M.I., Irizarry, R.A., Kingsford, C., 2017. Salmon provides fast and bias-aware quantification of transcript expression. Nat. Methods 14, 417–419. https://doi.org/10.1038/nmeth.4197

Priyanka, R., Vasundhara, M., Rao, G.G.E., Thara, B.S., Radhika, B., Marappa, N., 2017. Antioxidant activity of turmeric (Curcuma longa L.) cultivars. Med. Plants 9, 189–194. https://doi.org/10.5958/0975-6892.2017.00029.6

R Development Core Team 3.0.1., 2013. A Language and Environment for Statistical Computing. R Found. Stat. Comput.

Rasool, S., Geetha, T., Broderick, T.L., Babu, J.R., 2018. High Fat With High Sucrose Diet Leads to Obesity and Induces Myodegeneration. Front. Physiol. 9. https://doi.org/10.3389/FPHYS.2018.01054

Rastogi, R.P., Singh, S.P., Häder, D.P., Sinha, R.P., 2010. Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate in the cyanobacterium Anabaena variabilis PCC 7937. Biochem. Biophys. Res. Commun. 397, 603–607. https://doi.org/10.1016/j.bbrc.2010.06.006

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2009. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140. https://doi.org/10.1093/bioinformatics/btp616

Rui, L., Yuan, M., Frantz, D., Shoelson, S., White, M.F., 2002. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J. Biol. Chem. 277, 42394–42398. https://doi.org/10.1074/jbc.C200444200

Shahzadi, H., Khan, Z.H., Khan, N.D., Mular, S.M., 2017. Phytochemical screening of Holarrhena antidysenterica. Int. J. Appl. Res. 3, 560–561.

Sharma, D.K., Gupta, V.K., Kumar, S., Joshi, V., Mandal, R.S.K., Prakash, A.G.B., Singh, M., 2015. Evaluation of antidiarrheal activity of ethanolic extract of Holarrhena antidysenterica seeds in rats. Vet. world 8, 1392–5. https://doi.org/10.14202/vetworld.2015.1392-1395

Shiau, M.Y., Chiou, H.L., Lee, Y.L., Kuo, T.M., Chang, Y.H., 2017. Erratum to "Establishment of a Consistent L929 Bioassay System for TNF-α Quantitation to Evaluate the Effect of Lipopolysaccharide, Phytomitogens and Cytodifferentiation Agents on Cytotoxicity of TNF-α Secreted by Adherent Human Mononuclear Cells." Mediators Inflamm. 2017, 7327215. https://doi.org/10.1155/2017/7327215

Sinisgalli, C., Faraone, I., Vassallo, A., Caddeo, C., Bisaccia, F., Armentano, M.F., Milella, L., Ostuni, A., 2020. Phytochemical Profile of Capsicum annuum L. cv Senise, Incorporation into Liposomes, and Evaluation of Cellular Antioxidant Activity. Antioxidants 9, 428–440. https://doi.org/10.3390/antiox9050428

Soneson, C., Love, M.I., Robinson, M.D., 2016. Differential analyses for RNA-seq: Transcript-level estimates improve gene-level inferences [version 2; referees: 2 approved]. F1000Research 4, 1–22. https://doi.org/10.12688/F1000RESEARCH.7563.2

Tanvir, E.M., Hossen, M.S., Hossain, M.F., Afroz, R., Gan, S.H., Khalil, M.I., Karim, N., 2017. Antioxidant properties of popular turmeric (Curcuma longa) varieties from Bangladesh. J. Food Qual. 2017. https://doi.org/10.1155/2017/8471785

Tota, K., Rayabarapu, N., Moosa, S., Talla, V., Bhyravbhatla, B., 2013. InDiaMed : A Comprehensive Database of Indian Medicinal plants for Diabetes. Bioinformation 9, 378–380.

Tsalamandris, S., Antonopoulos, A.S., Oikonomou, E., Papamikroulis, G.A., Vogiatzi, G., Papaioannou, S., Deftereos, S., Tousoulis, D., 2019. The role of inflammation in diabetes:

Current concepts and future perspectives. Eur. Cardiol. Rev. 14, 50–59. https://doi.org/10.15420/ecr.2018.33.1

Upadhayay, V.K., 2016. Experimental approaches for determining various bioactivities of herbal plant extracts.

Verran, J., Redfern, J., Burdass, D., Kinninmonth, M., 2014. Using Soxhlet Ethanol Extraction to Produce and Test Plant Material (Essential Oils) for Their Antimicrobial Properties †. J. Microbiol. Biol. Educ. 15, 45–46. https://doi.org/10.1128/jmbe.v15i1.656

Volpe, C.M.O., Villar-Delfino, P.H., Dos Anjos, P.M.F., Nogueira-Machado, J.A., 2018. Cellular death, reactive oxygen species (ROS) and diabetic complications review-Article. Cell Death Dis. 9. https://doi.org/10.1038/s41419-017-0135-z

Yao, H.R., Liu, J., Plumeri, D., Cao, Y.B., He, T., Lin, L., Li, Y., Jiang, Y.Y., Li, J., Shang, J., 2011. Lipotoxicity in HepG2 cells triggered by free fatty acids. Am. J. Transl. Res. 3, 284–291.

Yu, G., Wang, L.G., Han, Y., He, Q.Y., 2012. ClusterProfiler: An R package for comparing biological themes among gene clusters. Omi. A J. Integr. Biol. 16, 284–287. https://doi.org/10.1089/omi.2011.0118

LIST OF PUBLICATIONS

1. **C. A. Divya,** Sharmila Lakshmi T. V, Smitha P. K, Sujan K Dhar, Manjula Shantaram, Manjula Das, "Antioxidant and antidiabetic activity of *Holarrhena antidysenterica*", Biomedicine, 2021; Suppl.41(2): 401-408. DOI: https://doi.org/10.51248/.v41i2.1046

2. **C. A. Divya**, Sujan K Dhar, Manjula Shantaram, Manjula Das, "Comparison of anti-diabetic effects of *Holarrhena antidysentrica* extracts with standard drugs: Results from a Longitudinal Meta-analysis of animal studies". bioRxiv preprint. February 12, 2021. doi: https://doi.org/10.1101/2021.02.11.430868

3. **C.A.Divya,** Sujan K Dhar, C. S. Anithkumar, Manjula Das, Manjula Shantaram "Comparison of Anti-diabetic, Anti-oxidant and Anti-lipogenesis activity of *Holarrhena antidysentica* plant parts".Medicinal Plants - International Journal of Phytomedicines and Related Industries. June 2022; Vol.14: 240-250. doi: 10.5958/0975-6892.2022.00027.2.

PRESENTED IN CONFERENCES

- **C. A. Divya** and Dr K. C. Pushpalatha Presented a review paper on "*Holarrhena antidysenterica* as Anti-Diabetic" at 8th National Women's science congress, held by Swadeshi Vijnana Andolan-Bangalore. November 2017, Karnataka, India
- **C. A. Divya**, Dr Manjula Shantaram, Dr Manjula Das, "*Holarrhena antidysenterica* as an anti-diabetic", 41st Annual International online Conference of Indian Association of Biomedical Scientists, organized by IABMS, March 2021, Karnataka, India.